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P450 in wild animals as a biomarker of environmental impact

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The impact of environmental pollution on selected animals was tested by monitoring the hepatic content of cytochromes P450 and their enzyme activities or by calculating TEQ values from the concentration of pollutants in the body. Fish-eating Stellars Sea Eagles, *Haliaeetus pelagicus*, found dead in the northern part of Hokkaido island accumulated high levels of PCBs and DDT and metabolites. The TEQ values calculated from the PCB concentration in the eagles were high enough to cause a significant toxic effect in other birds living in the same environment. Some of these birds were also contaminated with high concentrations of lead. Spotted seals, *Phoca largha*, captured along the coast-line of Hokkaido accumulated PCBs in their fat at about 100 million times the concentrations in the surface sea water. The levels of expressions of hepatic microsomal CYP 1A1and related enzyme activities in these seals showed good correlation to the levels of PCBs accumulated in the fat. The fresh water crabs, *Eriocheir japonicus*, were captured from three different rivers with various degrees of pollution. The P450 content and the related enzyme activities showed good correlation to TEQ values obtained from the concentrations of PCBs and PCDDs in the crabs from the rivers. The wild rodents, *Clethrionomys rufocanus*, were captured from urban, agricultural, and forest areas in Hokkaido. Those from the forest area had the lowest CYP content and related enzyme activities, comparable to those in laboratory-raised animals. Those from the urban areas, presumably contaminated with PAHs from fuel combustion, showed increased CYP 1A1 content and related enzyme activities. Those from the agricultural areas showed increased levels of CYP 1A1, 2B, 2E1. Rats treated with some of the agrochemicals used in the area resulted in a similar pattern of induction. It is concluded that P450 can be a useful biomarker for assessing the environmental impact of chemical pollutants on wild animals.

Keywords: P450, biomarker, Stellars Sea Eagle, seal, crab, PCDD, PCB, DDT, *Haliaeetus pelagicus*, *Phoca largha*, *Eriocheir japonicus*, *Clethrionomys rufocanus*.

Abbreviations: CYP, cytochrome P450; DDT, dichlorodiphenyltrichloroethane; EROD, ethoxyresorfin-*O*-deethylase; PCB, polychlorinated biphenyl; PCDD, polychlorinated dibenzodioxin; PCDF, polychlorinated dibenzofuran; TEF, toxic equivalence factor; TEQ, toxic equivalent.

Introduction

Biomarker(s) for assessing environmental impact of chemical pollutants on wild animals are urgently required. Identification and quantification of all the chemicals polluting the environment and assessing the risk is time consuming and costly. Furthermore, chemical analysis alone cannot predict enhancing or synergistic effects due to chemical mixtures present in the environment.

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Animals often respond to chemical exposure by inducing cytochrome P450s (CYPs), the major group of drug-metabolizing enzymes present in the liver. CYP metabolically detoxify a number of xenobiotics including drugs, chemical pollutants and agrochemicals. Since cytochrome P450 (CYP) 1A1 is an enzyme induced by a number of toxic xenobiotics (Snyder and Remmer 1982) the level and activity of this enzyme in the liver of wild animals may be a useful biomarker of environmental pollution.

Methods

Animals

Frozen samples of four fish-eating Stellars Sea Eagle, *Haliaeetus pelagicus*, found dead in northern parts of Hokkaido island were obtained from Shiretoko Museum. Thirteen spotted seals, *Phoca largha*, were hunted by the licensed hunter in a legally controlled programme. The liver samples and the fat tissues were obtained on the site. The wild rodents, *Clethrionomys rufocanus*, were captured from (i) a small bush in Sapporo City, representing an urban environment, (ii) a forest in Tobetu representing an agricultural environment, and (iii) a forest in Nakagawa, representing a rural forest environment. The fresh water crabs, *Eriocheir japonicas*, were captured from three different rivers, 8–16 crabs from each river. The Tone river runs through urban and industrial areas. the Barato river through suburban agricultural areas and the Shiribetu river through rural agricultural areas.

Determination of concentration of pollutants

Concentrations of PCBs (Tanabe *et al*. 1987), PCDDs and PCDFs (Hashimoto *et al*. 1995) and organochlorine pesticides (Tanabe *et al*. 1987) from the biological samples were determined according to the methods described in the literature.

Assay for CYP contents and related enzyme activities

Liver microsomes of the rodents were prepared and the contents of CYP were determined according to the method of Omura and Sato (1964). Midgut microsomes were prepared according to Ishizuka *et al*. (1998a). Hepatic microsomal contents of CYP 1A1, 2B1, 2B2, 2E1 and 2D2 were determined by the Western immuno-blotting method using a specific antibody to each CYP isozyme. Ethoxyresorfin-*O*-deethylase and bunitrolol 4-hydroxylase activities were determined according to the methods of Greenlee and Poland (1978) and Fujita *et al*. (1996) respectively.

Results

Two approaches to evaluate the impact of environmental pollutants on the animals living in different environments were tested by using CYPs as indicator enzymes. They were:

(1) Application of the concept of toxic equivalency factor (TEF), where each chemical of interest is assigned a factor that is derived mainly from the potency of a chemical to bind to Ah receptors or to induce CYPs. The TEF is expressed as a relative ratio with the potency of 2, 3, 7, 8 TCDD (TCDD) taken as unity (Ahlborg 1994). With this approach, we determined the concentrations of all the chemicals of interest in a biological sample used and the concentration of each chemical is multiplied by its TEF to determine the toxic impact in terms of TCDD-equivalent concentrations (toxic equivalent, TEQ). In the current study, this approach was applied to the fish-eating Stellars Sea Eagle, *Haliaeetus pelagicus*, found dead in northern parts of Hokkaido island and which has been shown to contain high concentrations of PCBs.

(2) Assessment of the CYP contents in the liver and related enzyme activities in animals inhabiting different environments. With this approach, if chemicals are present in high enough concentrations to exert their biological effect, then

induction of CYP could be expected. The induction of CYP may or may not be a result of synergism of two or more chemicals. The degree of induction of CYP may reflect the combined impact of environmental chemicals on the animals. This approach was applied to wild rodents, representing terrestrial animals, fresh water crabs (Ishizuka *et al*. 1996, 1998a), representing animals inhabiting fresh water, and seals, representing a species in the marine environment.

Contamination of Stellars Sea Eagles with PCBs and DDTs

As seen in table 1, fish-eating Stellars Sea Eagle, *Haliaeetus pelagicus*, found dead in northern parts of Hokkaido island, accumulated high levels of PCBs and DDT and its metabolites. These concentrations were comparable to those reported to be high concentrations in Bald Eagles at Lake Superior (Kozie and Anderson 1991) and White Tailed Eagles in the Baltic Sea region (Koistinen *et al*. 1995). The TEQ values calculated from the PCB concentration in the eagle were high enough to cause a significant toxic effect in other birds living in the same environment.

The satellite-assisted telemetric chase of the migrating eagles performed by the Japan Wild Bird Association with the technical help from Nippon Electric Co. (NEC) revealed that they nest on the Russian Pacific coast and migrate from there along the pacific coast down to Hokkaido, Japan in winter. The air and water of the Russian towns along the Pacific coast had several to tens of fold higher PCB concentrations than those in Hokkaido (Iwata *et al*. 1995) (table 2). Some of these birds were also contaminated with high concentrations (75–139 $\mu g\ g^{-1}$ liver) of lead (Pb), possibly due to ingestion of deer meat contaminated with the fragments of Pb bullets (Kim *et al*. 1999).

Contamination of seals with PCBs and DDTs and induction of cytochrome P450 1A1

Spotted seals, *Phoca largha*, travel along the coast-line of the north pacific to Hokkaido in winter. Since they also eat fish from the same area as the Stellars Sea Eagles, it was thought that they may accumulate similar pollutants in high

Table 1. Concentration of pollutants in breast muscle of Stellars Sea Eagles.

Pollutants	Concentration range ($ng\ g^{-1}$ wet wt)	TEQ (Ahlborg TEF) ($pg\ g^{-1}$)
PCBs	2000–20000	100–540
DDTs	2000–15000	

TEQ values were calculated using TEF proposed by Ahlborg *et al*. (1994) from the concentrations of PCBs in the breast muscle of Stellars Sea Eagle.

Table 2. Concentrations of PCB and DDT in the air from Magadan, Khabarovsk and Abashiri.

	Magadan	Khabarovsk	Abashiri
PCB	600	4900	200
DDT	690	240	10

Data from Iwata *et al*. (1995).

concentrations in their body. As seen in table 3, the concentrations of the PCBs and DDT in the fat of Stellars Sea Eagle were over 100 million and one billion times the concentrations found in the surface sea water.

The levels of expression of hepatic microsomal CYP 1A1 and some related monooxygenase activities in these seals showed good correlation to the level of PCBs in the fat and to the calculated TEQ values (figure 2). This suggests that the hepatic levels of CYP 1A1 in seals may be a useful biomarker of marine pollution.

Alterations of relative abundance of cytochrome P450 isozymes in wild rodents depending on their habitats

The wild rodents, *Clethrionomys rufocanus*, were captured from (i) a small bush in Sapporo City, representing an urban environment, (ii) a forest in Tobetu representing an agricultural environment and (iii) a forest in Nakagawa, representing a rural forest environment. The animals from the rural forest area showed the lowest levels of CYP isozymes and related enzyme activities comparable to those in laboratory-raised control animal. Those from the urban area, presumably contaminated with polycyclic aromatic hydrocarbons (PAHs), possibly from fuel combustion, showed increased CYP 1A1 content and related monooxygenase activities. CYP 2B and 2E1 were also increased, but not to the extent seen in the rodents from the agricultural area. Those from the agricultural

Table 3. Concentration of pollutants in body fat of spotted seals.

Pollutants	Concentration range (ng g^{-1} wet wt of fat)	TEQ (Ahlborg TEF) (pg g^{-1})
PCBs	300–4300	20–135
DDTs	300–5100	

The TEQ values were calculated from the concentrations of PCBs in the fat of the seals using TEF proposed by Ahlborg (1994).

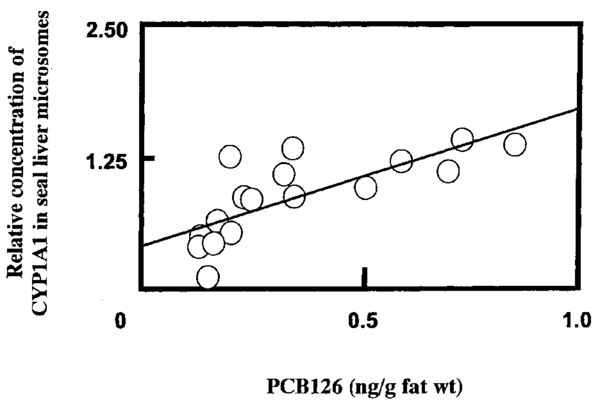

Figure 1. Relationships between CYP 1A1 contents and the concentrations of coplanar PCB in the blubber of the seal.

area, presumably contaminated with agrochemicals, showed high contents of CYP 1A1, 2B, 2E1.

Regardless of their origin, the xenobiotic-metabolizing activities of the animals were as low as those in the animals from forest area after a 3-month acclimatization in the laboratory with a standard rat diet. As can be seen in table 4, the rats experimentally exposed to some of the agrochemicals used in the agricultural areas (Ishizuka *et al*. 1998b), showed that agrochemicals can induce the same CYP isozymes found to be induced in *C. rufocanus* in the agricultural area.

Differences in cytochrome P450 activities in fresh water crabs and the degree of contamination of their origin

The fresh water crabs, *Eriocheir japonicas*, were captured from three different rivers. The Tone river runs through urban and industrial areas, the Barato river through suburban agricultural areas and the Shiribetu river through the rural agricultural areas. Crabs from all three rivers were contaminated with PCBs and PCDDs. TEQ values were calculated from the PCB and PCDD/PCDF concentrations in the midgut of crabs according to the TEFs formulated from bioassays using the Rainbow Trout cell line (Clemons *et al*. 1994, 1996). The crabs captured from the Tone river showed the highest P450 contents and related monooxygenase activities. The crabs from the Shiribetu river, the water of which is far less polluted, had the lowest P450 levels and related enzyme activities.

As can be seen in figure 2, the P450 content and the related enzyme activities showed good correlation to TEQ values calculated from the PCB and PCDD concentrations in the same crabs (Ishizuka *et al*. 1998a).

Discussion

Biomarkers which can assess the integrated impact caused by the multi-chemical pollution on the environment or on the animals in the environment are urgently needed. In this study, we tested the usefulness of P450 as a biomarker with two approaches: (1) application of the concept of TEQ and (2) direct measurement of P450 contents. TEQ values, calculated from PCB concentrations in Stellars Sea Eagles were high enough to warn of the danger of this already endangered species. The seals which are eating similar fish as the eagles in the same area showed high concentration of PCBs in their fat and the concentrations showed positive

Table 4. Effect of the living environment on the CYP contents in the liver of wild rodent species *c. rufocanus*.

	Induction of CYP (folds of control)			
	CYP 1A1	CYP 2B	CYP 2E1	CYP 3A
Forest area	1	1	1	1
Urban area	3–5	4	2–4	1–2
Agricultural area	3	6–8	8	1–2

C. rufocanus were captured from the sites indicated. Relative abundance of CYP isozymes were assessed by relative staining intensities of CYP bands in liver microsomes of *C. rufocanus* from different origin in Western blot analysis using corresponding anti-rat isozyme antibodies. Control: The staining intensities of liver microsomes from laboratory-raised animals.

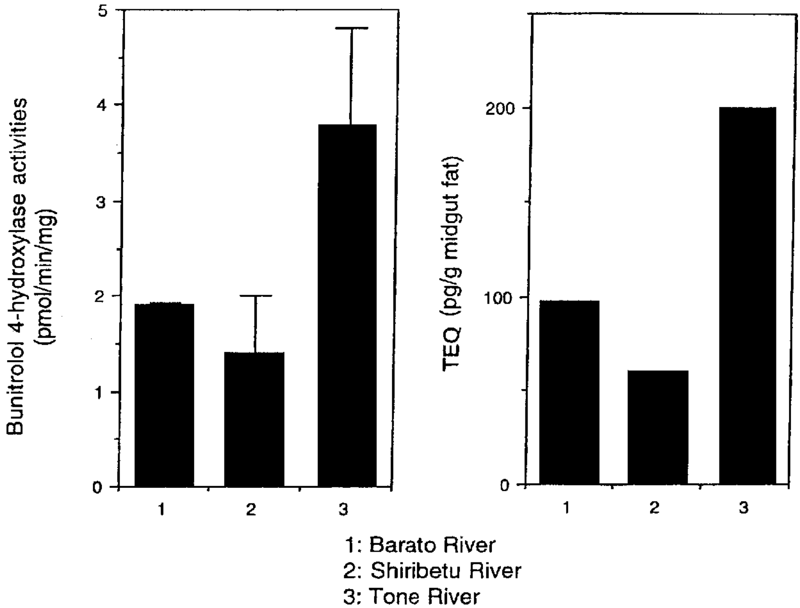

Figure 2. CYP-dependent drug-metabolizing activities in midgut microsomes of each captured from three different Japanese rivers and TEQ values in midgut fat of the same crabs.

correlation to the hepatic contents of cytochrome P450 1A1. The fresh water crabs from the polluted river showed high P450-related enzyme activities and high TEQ values as compared with the crabs from the less polluted river. Wild rodents from the urban area, presumably polluted with PAH, showed high hepatic P450 1A1 content, while those from the agricultural area showed high P450 1A1, 2B, 2E levels, which are shown to be induced by agrochemicals. Taken altogether it is concluded that P450 can be a useful biomarker for assessing the environmental impact of chemical pollutants on wild animals. The elevated P450 contents in the liver of wild animals have meant pollution of the environment where the animals are living. The measurement of P450 may be a useful pre-screening method for assessing environmental pollution before the costly chemical analysis.

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